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# Gas Chromatographic/Mass Spectrometric Differentiation of Atenolol, Metoprolol, Propranolol, and an Interfering Metabolite Product of Metoprolol

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16. Abstract

Pilots who successfully control their hypertension with medications, diet, and/or exercise can be medically certified to fly an aircraft. At the present time, approximately 8% of active pilots are designated as "hypertensive with medication" by the Federal Aviation Administration (FAA). One of the groups of antihypertensives is the beta-blocker. Of this group, atenolol, metoprolol, and propranolol are commonly prescribed, and they also have chemical and structural similarities. The FAA's Civil Aerospace Medical Institute (CAMI) toxicologically evaluates postmortem biological samples collected from pilots involved in fatal civil aviation accidents. Over the 10-year period, 1993-2002, CAMI has identified 50 pilot fatalities wherein atenolol was found in 24 pilots, metoprolol in 19 pilots, and propranolol in 7 pilots, which is consistent with the fact that these drugs have been in the lists of the top 200 most-prescribed drugs in the United States. In a few of the 50 pilot fatality cases, initial analysis suggested the presence of atenolol and metoprolol. However, there was no medical history with these cases supporting the use of both of these drugs, and it is also unusual for a patient to be prescribed atenolol together with metoprolol and/or propranolol. Therefore, further examination of the cases, wherein atenolol and metoprolol were apparently present, was undertaken. Atenolol, metoprolol, and/or propranolol, with their possible metabolite(s), were extracted from the selected case specimens, derivatized with pentafluoropropionic anhydride (PFPA), and analyzed by gas chromatography/mass spectrometry (GC/MS). The MS spectra of the PFPA derivatives of these 3 antihypertensives and a metoprolol metabolite are nearly identical. All of the PFPA derivatives had baseline GC separation, with the exception of a metoprolol metabolite product, which co-eluted with atenolol. There were 4 primary mass fragments (408, 366, 202, and 176 *m/z*) found with all of the PFPA-beta-blockers and with the interfering metabolite product. Therefore, this metabolite product could be misidentified as atenolol. However, atenolol has 3 unique fragments (244, 172, and 132 *m/z*), metoprolol has 2 unique fragments (559 and 107 *m/z*), propranolol has 4 unique fragments (551, 183, 144, and 127 *m/z*), and the metoprolol metabolite product has 2 unique fragments (557 and 149 *m/z*). These distinctive fragments were further validated by (i) using a computer program that predicts logical mass fragments and (ii) performing GC/MS of deuterated PFPA-atenolol and PFPA-propranolol and of the PFPA-alpha-hydroxy metabolite of metoprolol. By using the unique mass fragments, none of the re-examined pilot fatality cases were found to contain more than 1 beta-blocker. Several unique mass fragments reported in this study can be used for the positive identification of the 3 commonly used and chemically/structurally similar beta-blockers and a co-eluting interfering metabolite product of metoprolol. Therefore, these mass ions can be used for differentiating and simultaneously analyzing these beta-blockers in biological samples.

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# Gas Chromatographic/Mass Spectrometric Differentiation of Atenolol, Metoprolol, Propranolol, and an Interfering Metabolite Product of Metoprolol

## INTRODUCTION

Pilots who successfully control their hypertension with medication, diet, and/or exercise can be medically certified to fly aircraft. However, these pilots are considered hypertensive and are closely monitored by the Federal Aviation Administration (FAA) to ensure that their hypertension is properly controlled. Approximately 8% of the active pilots fall under the category of "hypertensive with medication." During the investigation of fatal civil aviation accidents, postmortem samples obtained from pilots are submitted to the FAA's Civil Aerospace Medical Institute (CAMI) for toxicological evaluation (1). During such evaluation, submitted samples are analyzed for prescription and nonprescription drugs (2), and it is common to find beta-blocker antihypertensives such as atenolol, metoprolol, and propranolol in the submitted biological samples. This observation is consistent with the fact that 28.7% of the U.S. general population have been diagnosed with hypertension (3), and these 3 antihypertensives have been in the lists of the top 200 drugs dispensed in the U.S. (4, 5).

During a 10-year period of 1993-2002, postmortem samples from 3290 civil aviation accident pilot fatalities (cases) were received by CAMI. Toxicological evaluation of these cases revealed that 50 of the 3290 fatalities had the commonly prescribed beta-blockers, atenolol, metoprolol, and propranolol. Out of the 50 fatalities, atenolol, metoprolol, and propranolol were found to be present in 24, 19, and 7 fatalities, respectively, but the initial analysis indicated the presence of atenolol and metoprolol in 4 of these pilot fatalities. Since (i) the combined use of both drugs was not consistent with the history of the drug use by those pilots, (ii) it is uncommon to simultaneously prescribe 2 beta-blockers, and (iii) these commonly used antihypertensives have considerable amount of chemical and structural similarity (6), further examination was undertaken for those fatality cases wherein atenolol and metoprolol were initially detected. Such examination entailed selectively and simultaneously analyzing the 3 commonly used beta-blockers in the submitted biological samples and rectifying any possible analytical interference with the antihypertensives.

## MATERIALS AND METHODS

### Materials

All chemicals, reagents, and solvents were purchased from commercial sources in the highest possible purity and were used without any further purification. Specifically, the beta-blockers atenolol, metoprolol, and propranolol were purchased from Sigma Chemical Company (St. Louis, MO); *N*-methyl-4-(4-bromophenyl)-1,2,3,4-tetrahydro-1-naphthylamine (internal standard) was purchased from Pfizer, Inc. (Groton, CT); pentafluoropropionic anhydride (PFPA) was supplied by Pierce, Inc. (Rockford, IL); and atenolol-$D_7$ and propranolol-$D_5$ were obtained from Cerilliant Corporation (Round Rock, TX). The alpha-hydroxy metabolite of metoprolol was kindly provided as a gift by AstraZeneca Pharmaceuticals (Macclesfield, Cheshire, UK). Bovine whole blood was obtained from Mikkelson Beff, Inc. (Oklahoma City, OK).

All aqueous solutions of drugs, chemicals, and reagents were prepared in double deionized water (DDW) obtained from Milli-$QT_{plus}$ Ultra-Pure Reagent Water System (Millipore®, Continental Water Systems, El Paso, TX). A 500-ng/mL solution of the internal standard was also prepared in DDW. Positive controls of the beta-blockers at a concentration of 200 ng/mL were prepared in bovine whole blood from their 1.0 mg/mL methanolic standards. These controls were prepared in pools large enough to provide replicates for the entire study. Bovine blood was also used for negative controls. All samples were stored at –20°C until analyzed.

### Extraction

Solid tissue specimens were homogenized in DDW (1:2 w/w) by using a PRO250 post-mounted homogenizer (Pro Scientific, Oxford, CT), equipped with a generator (10 mm diameter) set at 22,000 rpm. Three-mL aliquots of controls and specimen fluids, and 3.0-g aliquots of tissue homogenates were transferred to individual 16 × 150 mm screw top glass test tubes, and 1.0 mL (500 ng/mL) of the internal standard solution was added to each tube. Mixtures in the tubes were vortexed briefly and allowed to stand for 10 min. To each of the tubes, except those containing urine, was added 9.0 mL of ice-cold aceto-

nitrile (4°C), and the contents in the tubes were mixed on a rotary mixer for 15 min. Subsequently, the tubes were centrifuged at 820 × *g* for 5 min to remove cellular particulate matter, including precipitated protein. The supernatants in the tubes were then transferred to 16 × 100 mm culture tubes and evaporated to less than 1 mL in a 40°C-water bath under a stream of dry nitrogen. To all the tubes, including the tubes that contained urine samples, was then added 4.0 mL of 0.10 M phosphate buffer (pH 6.00).

The buffered mixtures were transferred to solid-phase extraction (SPE) columns (Bond Elute Certify® Columns, Varian Co., Harbor City, CA), which were pre-conditioned with 2.0 mL methanol, followed by 2.0 mL of the 0.10 M phosphate buffer. A column flow rate of 1–2 mL/min was maintained in each step by using a Varian 24 port positive pressure manifold (Varian Co., Harbor City, CA) with approximately 3 psi of nitrogen. Once the extracts had passed through, the columns were washed with 1.0 mL of 1.0 M acetic acid, followed by 6.0 mL of methanol. The columns were dried after each wash for 5 min with 25-psi nitrogen. Analytes were eluted off the columns with 4.0 mL of the freshly prepared 2% ammonium hydroxide in ethyl acetate, and the eluants were collected in conical glass test tubes.

The eluants collected in the test tubes were then evaporated to dryness in a water bath at 40°C under a stream of dry nitrogen, and subsequently, PFPA (50.0 µL) and ethyl acetate (50.0 µL) were added to each tube. These tubes were then capped, vortexed briefly, and incubated in a heating block set to 70°C for 20 min for the PFPA-derivatization. After removing the tubes from the heating block and allowing them to cool to ambient temperature, the reaction mixtures were evaporated to dryness in the 40°C water bath. The residues were then reconstituted in 50.0 µL of ethyl acetate and transferred to autosampler vials for analyses.

### Gas Chromatographic/Mass Spectrometric Conditions

Analyses were performed by using a bench-top gas chromatograph/mass spectrometer (GC/MS) system, consisting of a Hewlett Packard (HP) 6890 series GC, interfaced with a HP 5973 quadrupole MS (Agilent Technologies, Palo Alto, CA). The GC/MS system was operated with a transfer line temperature of 280°C and a source temperature of 230°C. The GC separation of the analytes was achieved by using a HP-ULTRA-1 crosslinked 100% methyl siloxane capillary column (12 m × 0.2 mm i.d., 0.33 µm film thickness; Agilent Technologies, Palo Alto, CA). Helium was employed as the carrier gas at a flow rate of 1.0 mL/min. A HP 6890 autosampler was used to inject 1.0 µL of PFPA-derivatized products in ethyl acetate onto the GC/MS system. The GC was equipped with a split/splitless injection port operated at 250°C in the splitless mode with the purge time of 0.5 min. The GC oven temperature profile was 70–290°C at 30°C/min, with a final hold time of 2.67 min, resulting in a total run time of 10 min. Initially, PFPA-derivatized standards of each analyte (1.0 µL of a 100.0 ng/µL ethyl acetate solution) were injected individually and analyzed using the full MS scan mode of 40 to 800 atomic mass units. Subsequently, the MS was operated in selected ion monitoring mode with a dwell time of 30 msec.

### Analysis Acceptability Criteria

Mass ions used for the identification and confirmation of each analyte were selected based on their abundance, mass-to-charge ratio (*m/z*), and uniqueness (Table 1). Acceptability criteria employed for the analysis were (i) ion

**Table 1**. Retention times and mass fragments of the beta-blockers and interfering metabolite product.

| Analytes | Retention Times (min) | Common Ions (m/z) | Unique Ions (m/z) |
|---|---|---|---|
| Atenolol | 5.74 | 408, 366, 202, 176 | 244,172,132 |
| Metoprolol | 5.50 | | 559,107 |
| Propranolol | 5.80 | | 551,183,144,127 |
| Interfering metoprolol metabolite product | 5.75 | | 557,149 |

ratios for a given analyte, measured as the peak area of a qualifier ion divided by the peak area of the confirmation ion, were required to be within ± 20% of the average of the ion ratios for the respective controls analyzed during that analysis; (ii) each ion monitored was required to have a minimum signal-to-noise ratio of 5; and (iii) the analyte was required to have a retention time within ± 0.20 min of the average retention time for the respective controls analyzed during that analysis. Any analysis of a particular analyte not meeting the aforementioned criteria was considered as either being negative or inconclusive for the analyte.

**Mass Fragment Prediction**

The distinctive fragments chosen for each analyte were validated for their uniqueness by using a computer program that predicted logical mass fragments of chemical compounds (HighChem Mass Frontier™, ThermoFinnigan Corp., San Jose, CA). The predicted mass fragments were also subsequently confirmed by using the mass fragment patterns of the PFPA-derivatized atenolol-$D_7$, propranolol-$D_5$, and alpha-hydroxymetoprolol.

## RESULTS AND DISCUSSION

The re-examination of the full scan mass spectral data of those fatality cases, wherein atenolol and metoprolol were initially suspected, revealed that the apparent atenolol peak was not atenolol, but it was most likely a co-eluting metabolite product of metoprolol. Such near misidentification highlighted a need for the development of a selective analytical method that can differentiate commonly used, chemically/structurally similar beta-blockers (6) and their possible interfering metabolite(s)/ product(s). In the present study, a GC/MS method is described that selectively distinguishes the 3 beta-blockers from each other and from an interfering metabolite product of metoprolol.

Under the adopted instrumental conditions, the retention times for metoprolol, atenolol, and propranolol were 5.50, 5.74, and 5.80 min, respectively (Table 1), suggesting a baseline separation (Fig. 1). However, a metoprolol metabolite product interfered with atenolol, as this product co-eluted with the beta-blocker. The mass spectra of these 4 PFPA-analytes were nearly identical, as there were 4 dominating common fragments (408, 366, 202, and 176 m/z) with the 3 beta-blockers and the interfering metabolite product (Figs. 2-5). Given the retention times and the 4 dominating mass fragments, the metoprolol metabolite product could easily be misidentified as atenolol. This limitation was the reason for initially suspecting both atenolol and metoprolol in the pilot fatality cases. However, further in-depth examination of the mass spectral characteristics and chemical fragments of the analytes revealed the presence of 3 unique fragments (244, 172, and 132 *m/z*) for atenolol, 2 unique fragments (559 and 107 *m/z*) for metoprolol, 4 unique fragments (551, 183, 144, and, 127 *m/z*) for propranolol, and 2 unique fragments (557 and 149 *m/z*) for the interfering metoprolol metabolite product. The uniqueness of the mass ions was further confirmed by the ThermoFinnigan HighChem Mass Frontier™ computer program and by the PFPA-atenolol-$D_7$, PFPA-propranolol-$D_5$, and PFPA-alpha-hydroxymetoprolol GC/MS analyses.

The 3 major urinary metabolites of metoprolol are *O*-demethylated and oxidized, oxidative-deaminated, and aliphatic hydroxylated products (7, 8). The first 2 metabolites have a carboxyl group and a secondary

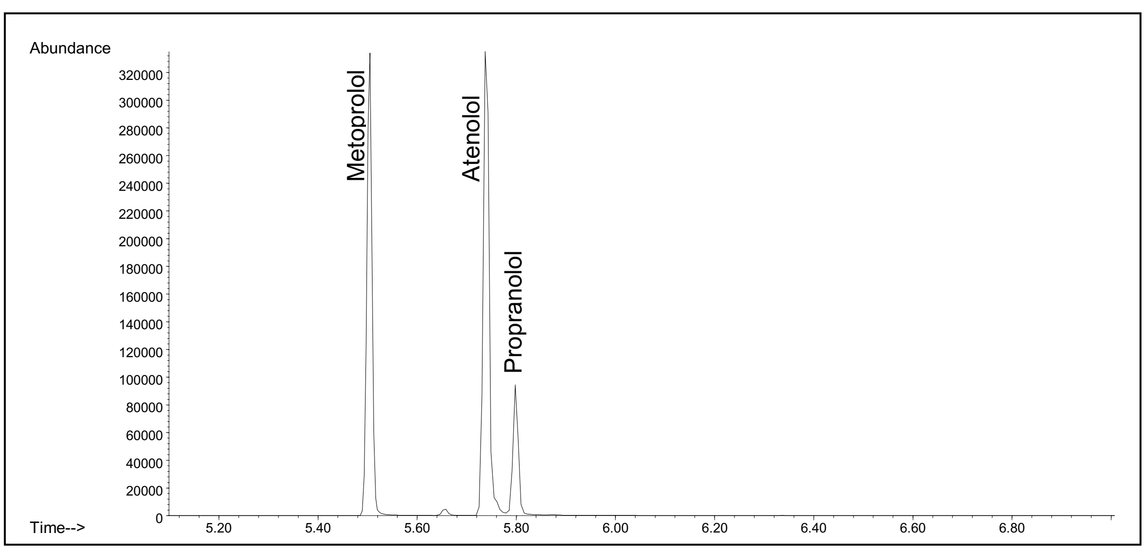


**Figure 1.** Total ion chromatogram of the PFPA-derivatized atenolol, metoprolol, and propranolol.

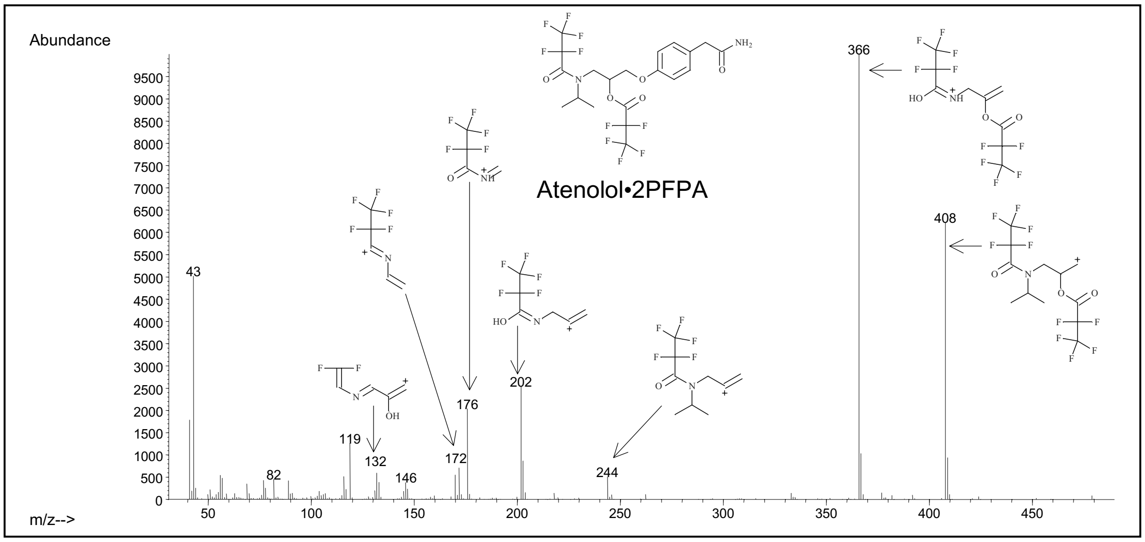


**Figure 2.** PFPA-atenolol mass spectrum, showing unique mass fragments for atenolol and mass fragments shared by all 3 beta-blockers and by the interfering metoprolol metabolite product.

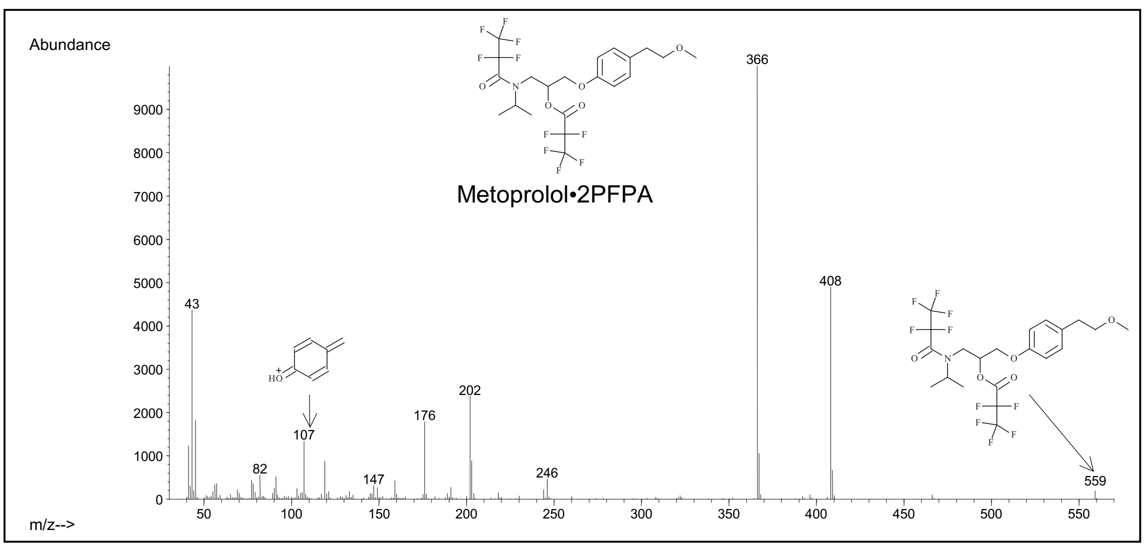


**Figure 3.** The mass spectrum of the PFPA-derivatized metoprolol, along with the chemical structures of the derivatized beta-blocker and primary fragments.

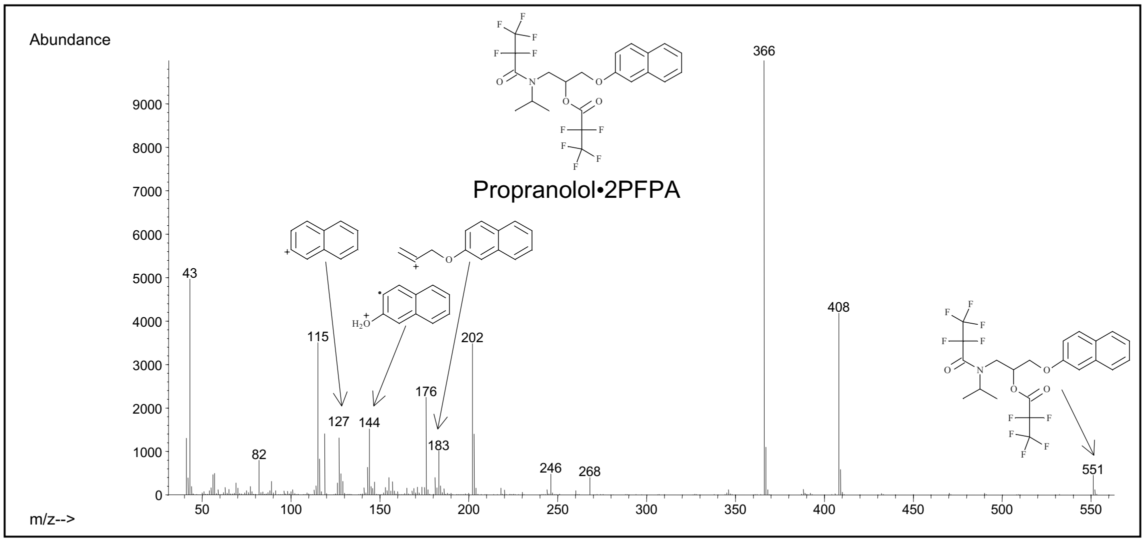


**Figure 4.** The mass spectrum of the PFPA-derivatized propranolol, along with the chemical structures of the PFPA-propranolol and relevant mass fragments.

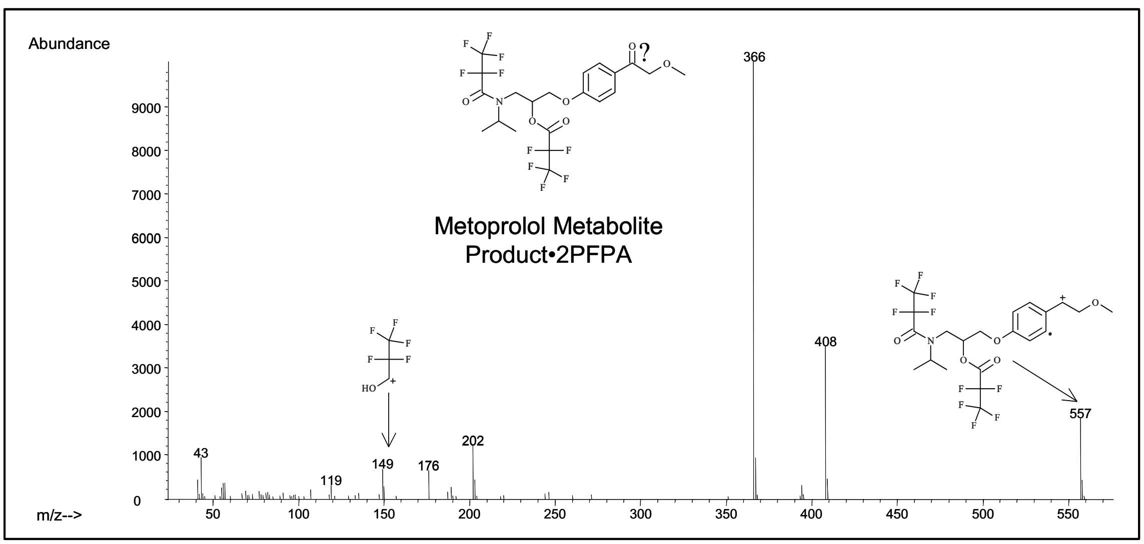


**Figure 5.** The mass spectrum of the product generated from the PFPA-derivatized alpha-hydroxymetoprolol, along with the likely chemical structures and primary fragments.

aliphatic hydroxyl group, but 1 of these 2 metabolites also has a secondary amino group. The third metabolite has 2 aliphatic hydroxyl groups and 1 secondary amino group. Based on the extraction and GC procedures adopted in this study and the selected mass fragment pattern of the interfering peak, the 2 acid metabolites were ruled out as possible interfering substances with atenolol. The third hydroxyl and amino group containing metabolite (alpha-hydroxymetoprolol) has 3 sites that can react with PFPA and, thereby, can generate a product with 3 $CF_3CF_2CO$– moieties. Approximately 0.11 min prior to the atenolol-interfering peak, there was also a non-interfering peak that had a parent ion and a mass fragmentation pattern consistent with that expected for the $(CF_3CF_2CO)_3$–hydroxyl/amino metoprolol metabolite, i.e., alpha-hydroxymetoprolol, but the analyte that co-eluted with atenolol had mass fragments similar to those of a $(CF_3CF_2CO)_2$–hydroxyl/amino metoprolol product, having a keto group in place of the hydroxyl group of alpha-hydroxymetoprolol. The presence of the $(CF_3CF_2CO)_2$–metoprolol product could not be explained by the non-derivatization of the alpha-hydroxyl site, since there is no apparent steric hindrance for the site in the molecule. However, it could be possible that the hydroxyl group would have been converted into the keto group because of a chemical rearrangement prior to, or during, the PFPA derivatization and/or the subsequent loss of 1 of the 3 $CF_3CF_2CO$– groups of the derivatized alpha-hydroxymetoprolol upon exposure to the high temperature at the GC injector port, thus also generating a product with only 2 $CF_3CF_2CO$– groups and a keto group. The mass spectral characteristics of this interfering metabolite product (Fig. 5) were further confirmed by analyzing a standard of alpha-hydroxymetoprolol after its PFPA derivatization. During this analysis, the interfering metabolite product peak was present along with the peak of 3 PFPA-alpha-hydroxymetoprolol. These findings suggest that the alpha-hydroxy metabolite of metoprolol was partially converted to the interfering, possible keto product.

While atenolol was initially identified in a few metoprolol-positive cases, it was not detected in all metoprolol cases. This finding is most likely because the formation of the interfering metabolite product seems to be dependent upon the concentration of alpha-hydroxymetoprolol. In cases where alpha-hydroxymetoprolol was present in small quantities, the interfering product was undetectable and, thus, was not found to contain the atenolol-interfering analyte. Because of this concentration-production relationship, atenolol was initially detected or suspected in only a few metoprolol-positive cases wherein alpha-hydroxymetoprolol was possibly present in large quantities.

Overall, several unique mass fragments reported herein can be used to positively identify the 3 common beta-blockers and the co-eluting metoprolol metabolite product. By monitoring the selected unique mass fragments and, thereby, taking the selective analytical approach, none of the mentioned pilot fatality cases were found to contain more than 1 beta-blocker. Therefore, the analytical method reported in this study can be suitably adopted for selectively differentiating and simultaneously analyzing these structurally similar beta-blockers in biological samples.

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